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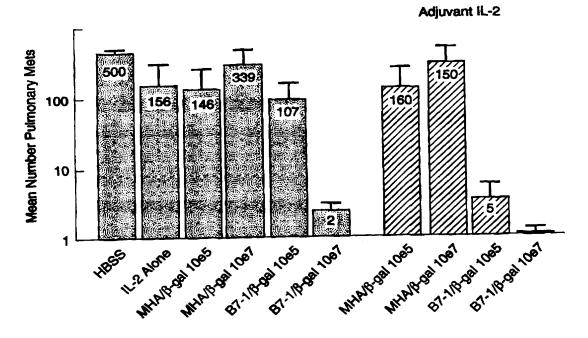| 08/317,402 | 3 October 1994 (03.10.94) | US |
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(57) Abstract

The present invention is a recombinant virus which has incorporated into its genome or portion thereof a gene encoding an antigen at a disease causing agent in combination with an immunostimulatory molecule for the purpose of stimulating an immune response against the disease causing agent. Methods of treatment of diseases such as cancer and diseases caused by pathogenic microorganisms are provided using the recombinant virus.

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ENHANCED IMMUNE RESPONSE
BY INTRODUCTION OF CYTOKINE GENE AND/OR
COSTIMULATORY MOLECULE B7 GENE IN A
RECOMBINANT VIRUS EXPRESSING SYSTEM

FIELD OF THE INVENTION

The present invention relates to recombinant viral vector vaccines for the prevention or treatment of pathogenic diseases and cancer. More particularly, it relates to recombinant viral vector vaccines comprising a genes encoding an antigens and a gene(s) encoding an immunostimulatory molecule(s).

BACKGROUND OF THE INVENTION

The five year survival of patients with metastatic melanoma is less than 2% in most reported series. Combination chemotherapy can induce objective regressions of melanoma but this treatment is rarely, if ever, curative. Tumor infiltrating lymphocytes (TIL) have been identified in patients with melanoma that appear to recognize unique cancer antigens in an MHC restricted fashion. (Rosenberg, S.A. J. Clin. Oncol. 10:180-199, 1993; Topalian, S.C. et al Tumor-infiltrating lymphocytes: Evidence for specific immune reactions against growing cancers in mice and humans. In: De Vita, Hellman and Rosenberg (eds) Important Advances in Oncology, Philadelphia, J.B. Lippincott Co., 1990, pp 19-41, Schwartzentruber et al J. Immunol 146:3674-3681, 1991). The adoptive transfer of TIL can mediate regression in 35 to 40% of patients with advanced melanoma and studies are underway to attempt to generate more potent TIL by genetic modification of TIL or by immunization with preparations containing tumor associated antigens. Rosenberg, J. Clin. Oncol. 10:180-199, 1993; Rosenberg, S.A. J. Am. Med. Assoc 268:2416-2419, 1992.

A large number of studies in experimental animals have demonstrated that the cellular rather than the humoral arm of the immune response plays the major role in the elimination of murine tumors. Wunderlich, J.R. et al.

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Principles of tumor immunity: In: DeVita et al (eds) Biologic Therapy of Cancer, Philadelphia: J.B. Lippincott Co. 1991, pp 3-21. Much of this evidence was derived from studies in which the adoptive transfer of T lymphocytes from immune animals could transfer resistance to tumor challenge or in some experiments the actual elimination of established cancer. Thus, most strategies for the immunization of patients with cancer have been directed at stimulating strong T cell immune reactions against tumor associated antigens.

Most attempts at active immunization against cancer antigens have involved whole tumor cells or tumor cell fragments, though it would be most desirable to immunize specifically against unique tumor antigens that distinguish malignant from normal cells. The molecular nature of the tumor associated antigens recognized by T lymphocytes is poorly understood. In contrast to antibodies that recognize epitopes on intact proteins, T cells recognize short peptide fragments (8-18 amino acids) that are presented on cell surface class I or II major histocompatibility (MHC) molecules and it is likely that tumor associated antigens are presented and recognized by T cells in this fashion.

A number of genes have been identified that encode melanoma tumor antigens recognized by TIL in the context of the HLA-A2 class I molecule. Kawakami, T. et al Proc. Nat'l Acad. Sci. 91:3515-3519, 1994; Kawakami, Y. et al J. Exp. Med. 180:347-352, 1994; Kawakami et al Cancer Res. 54:3124-3126, 1994. These antigens appear to be the most clinically relevant antigens responsible for mediating tumor regression in patients with advanced melanoma since the TIL used to identify these antigens were capable of mediating in vivo antitumor regression. Two such antigens, which appear to be present in virtually all fresh and cultured melanomas, have been called MART-1 (Melanoma Antigen Recognized by T Cells - 1) and gp100.

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The genes encoding both of the peptides have been cloned
and sequenced. The MART-1 gene encodes a 118 amino acid
protein of 13 kD. The gp100 gene encodes a protein
identical to that recognized by monoclonal antibody HMB-
45. With the exception of melanocytes and retina no
normal tissues express this antigen and no expression of
these gene products has been seen on cancers other than
melanoma. Both antigens, therefore, appear to be
melanocyte lineage specific.

The MART-1 antigen was expressed on all nine tissue
culture lines tested (that were established from melanomas
in the Surgery Branch, NCI) and on all fresh melanomas
tested. Studies by others showed that the MART-1 (also
called Melan-A) antigen was expressed on 26 of 26 fresh
melanomas. Coulie, P.G. et al J. Exp. Med. 180:35-42,
1992. The gp100 antigen is also widely expressed in
melanomas. In one study, reactivity with antibody HMB-45
(reactive with gp100) was present on 100% of non-spindle
cell type melanomas and on 62 of 67 total melanomas.
Wick, M.R. et al J. Cutan. Pathol. 4:201-207, 1988. In
another study, 32 of 35 melanomas studied (91%) expressed
gp100 (Ordonez, N.G. et al Am. J. Clin. Pathol. 4:385-390,
1988) and in a third study of 60 of 62 (97%) melanomas
expressed gp100 (Gown, A.M. et al Am. J. Pathol. 123:195-
203, 1986).

Of 14 separate TIL cells that were raised in the
Surgery Branch, NCI from different HLA-A2 individuals, 13
of 14 recognized MART-1 and 4 of 14 recognized the gp100
antigen. Because TIL cells that recognize these
determinants have been shown to be capable of mediating
cancer regression in vivo, it appears that these antigens
are involved in cancer regression.

Another gene coding for a human tumor specific
antigen on a human melanoma was cloned by Van der Bruggen
et al (Science 254:1643-1647, 1991). This antigen is
coded for by a gene called MAGE-1 which spans five

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kilobases. A 2,419 base pair coding sequence produces a
predicted protein product of 26 kD. The MAGE antigen is
HLA-A1 restricted and the nine amino acid fragment that
represents the A1 restricted immunodominant peptide has
been defined as Glu-Ala-Asp-Pro-Thr-Gly-His-Ser-Tyr. This
nine amino acid peptide is encoded by the third exon of
the MAGE gene. Transfection of a 500 base pair fragment
of this gene can confer recognition by a MAGE specific CTL
clone. Incubation of an EBV cell line with the
immunodominant peptide can confer sensitivity to lysis by
a MAGE-1 specific CTL clone. MAGE-1 does not appear to be
expressed in normal cells with the possible exception of
testis, but is expressed on approximately half of
metastatic melanomas, about 20% of breast cancers as well
as other selected types of cancer.

The identification of an immunodominant peptide that
represents a unique tumor antigen has opened new
possibilities for immunization against cancer.
Substantial evidence exists in animal models that
immunization with immunodominant viral peptides can induce
viral specific CTL that can confer protection against
viral infection. Although pure peptide alone is
ineffective in stimulating T cell responses, peptides
emulsified in adjuvants or complexed with lipids have been
shown to prime mice against challenge with fresh virus and
can induce virus specific CTL that protect mice against
lethal viral inocula (Kast, W.M. et al _Proc. Nat'l Acad._
Sci. U.S.A. 88:2283-2287, 1991; Deres, K. et al _Nature_
342:561-564, 1989; Gao, X.M. et al _J. Immunol._ 147:3268-
3273, 1991; Aichele, P. _J. Exp. Med._ 171:1815-1820, 1990;
Collins, D.S. et al _J. Immunol._ 148:3336-3341, 1992).
Immunization of mice against splenocytes coated with
Listeria _monocytogenes_ peptide epitopes also results in
the generation of Listeria specific CTL which can be
expanded in culture. Adoptive transfer of these CTL can
protect mice against lethal bacterial challenge (Harty,

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J.T. et al <u>J. Exp. Med.</u> 175:1531-1538, 1992). Peptides
representing antigenic epitopes of HIV gp120 and gp160
emulsified in complete Freund's adjuvant can also prime
specific CTL responses (Takahashi, H. et al <u>Proc. Nat'l</u>
<u>Acad. Sci. U.S.A.</u> 85:3105-3109, 1988; Hart, M.K. et al
<u>Proc. Nat'l Acad. Sci. U.S.A.</u> 88:9448-9452, 1991).

While immunization with peptides in adjuvants or
complexed with lipids gives rise to T cell responses in
mice, the reactions are rarely strong enough to induce T
reactive cells in primary splenocytes. The detection of
sensitized lymphocytes almost invariably requires
secondary <u>in vitro</u> stimulation.

The expression of the B7 gene family has been shown
to be an important mechanism of antitumor responses in
both mice and humans. It is now becoming apparent that at
least two signals are required for activation of naive T-
cells by antigen bearing target cells: an antigen specific
signal, delivered through the T-cell receptor, and an
antigen independent or costimulatory signal leading to
lymphokine products (Hellstrom, K.E. et al. <u>Annals NY</u>
<u>Acad. Sci.</u> 690:225-230, 1993). Two important
costimulatory molecules are B7-1, which is the ligand for
T-cell surface antigens CD28 and CTLA4 (Schwartz, R.H.
<u>Cell</u> 71:1065-1068, 1992; Chen, L. et al. <u>Cell</u> 71:1093-
1102, 1992; Freeman, G.J. et al. <u>J. Immunol</u> 143:2714-2722,
1989; Freeman, G.J. et al. <u>J. Exp. Med.</u> 174:625-631,
1991), and B7-2, an alternative ligand for CTLA4 (Freeman,
G.J. et al. <u>Science</u> 262:813-960, 1995). To date, both
murine B7-1 and B7-2 (Freeman, G.J. et al. <u>J. Exp. Med.</u>
174:625-631, 1991; Freeman, G.J. et al. <u>Science</u> 262:813-
960, 1995) and human B7-1 and B7-2 have been described
(Freeman, G.J. et al. <u>J. Immunol</u> 143:2714-2722, 1989;
Freeman, G.J. et al <u>Science</u> 262:909-911, 1993). It is
unclear at this time whether the costimulatory signals
provided by B7-1 and B7-2 are functionally distinct or
redundant mechanisms for T-cell activation (Hathcock, K.S.

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et al. J. Exp. Med. 180:631-640, 1994). Most murine and
human tumors do not express B7-1 or B7-2, implying that
even when a tumor expresses a potential rejection antigen,
it is unlikely to activate antitumor T-cell responses
(Hellstrom, K.E. et al Annals. N.Y. Acad. Sci. 690:225-
230, 1993); Hellstrom, I. Annals. N.Y. Acad. Sci. 690:24-
31, 1993). In essence, anergy may result from only one
signal being received by the T-cell (Hellstrom, K.e. et
al. Annals. N.Y. Acad. Sci. 690:225-230, 1993.
Transfection of B7 into melanoma cells was found to induce
the rejection of a murine melanoma in vivo (Townsend, S.E.
et al Science 259:368-370, 1993).

Vaccinia viruses have been extensively used in humans
and the use of a vaccina based vaccine against smallpox
has led to the worldwide eradication of this disease
(reviewed in reference Moss, B. Science 252:1662-1667,
1991). Vaccinia viruses have the advantages of low cost,
heat stability and a simple method of administration.
Attempts have been made to develop vaccinia virus vectors
for the prevention of other diseases.

Vaccina virus is a member of the pox virus family of
cytoplasmic DNA viruses. DNA recombination occurs during
replication of pox viruses and this has been used to
insert DNA into the viral genome. Recombinant vaccina
virus expression vectors have been extensively described.
These vectors can confer cellular immunity against a
variety of foreign gene products and can protect against
infectious diseases in several animal models. Recombinant
vaccina viruses have been used in human clinical trials as
well. Cooney et al immunized 35 healthy HIV seronegative
males with a recombinant vaccinia virus expressing the
gp160 envelope gene of HIV (Cooney, E.. The Lancet
337:567-572, 1991). Graham et al randomized 36 volunteers
to receive either recombinant vaccinia virus containing
the gp160 HIV envelope protein or control vaccinia virus
(Graham, B.S. et al J. Infect. Dis. 166:244-252, 1992).

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Phase I studies using recombinant vaccinia virus have
begun in patients with metastatic melanoma using a
recombinant virus expressing the p97 melanoma antigen
(Estin, C.D. et al *Proc. Nat'l Acad. Sci.* 85:1052-1056,
1988) and a trial to use recombinant vaccinia virus
expressing the human carcinoembryonic antigen in patients
with advanced colorectal carcinoma is about to begin
(Schlom, J. personal communication). In these trials,
vaccinia virus is administered by intradermal
scarification and side effects have been minimal including
local skin irritation, lymphadenopathy and transient flu-
like symptoms.

    Fowlpox viruses are members of the pox virus family
(avipox virus genes). Fowlpox virus will only replicate
in avian cells and cannot replicate in human cells. It is
a cytoplasmic virus that does not integrate into the host
genome but is capable of expression of a large number of
recombinant genes in eukaryotic cells.

    Recombinant fowlpox virus expressing rabies
glycoprotein has been used to protect mice, cats and dogs
against live rabies virus challenge. Immunization of
chickens and turkeys with a recombinant fowlpox expressing
the influenza HA antigen protected against a lethal
challenge with influenza virus (Taylor, J. et al *Vaccine*
6:504-508, 1988). Canarypox virus, another member of the
avipox genus similar to fowlpox, was safely administered
subcutaneously to 25 normal human volunteers at doses up
to 10" infectious doses (Cadox, M. et al *The Lancet*
339:1429-1432, 1992). In a recent trial sponsored by the
NIAID (Protocol 012A: A Phase I safety and immunogenicity
trial of live recombinant canarypox-gp160 MN (ALVAC VCP125
HIV-1gp160MN0 in HIV-1 uninfected adults) patients
received recombinant canarypox virus containing the HIV
gp160 gene by intramuscular injection at doses up to 10
pfu with little to no toxicity (personal communication, P.
Fast, NIAID).

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Fowlpox virus thus represents an attractive vehicle for immunization since it can stimulate both humoral and cellular immunity, it can be economically produced in high titer (10^9 pfu/ml) and yet its inability to productively infect human cells substantially increases the safety of its use, compared to replicating viruses such as vaccina view, especially in immunocompromised hosts.

Another considerable advantage of fowlpox virus is that there is apparently little or no cross-reactivity with vaccinia virus and thus previously vaccinated humans will not have pre-existing immune reactivity to fowlpox virus proteins.

SUMMARY OF THE INVENTION

The present invention is a recombinant virus comprising a viral genome or portion thereof, one or more nucleic acid sequences encoding one or more antigens of a disease causing agent and one or more nucleic acid sequences encoding one or more immunostimulatory molecules.

The present invention is also a composition comprising a recombinant virus comprising a viral genome or portion thereof and one or more nucleic acid sequences encoding one or more antigens of a disease causing agent and optionally an exogenous immunostimulatory molecule, chemotherapeutic drug, antibiotic, antifungal drug, antiviral drug, or combination thereof.

Another aspect of the present invention is a composition comprising a recombinant virus comprising a viral genome or portion thereof, one or more nucleic acid sequences encoding one or more antigens of a disease causing agent and one or more nucleic acid sequences encoding one or more immunostimulatory molecules and optionally an exogenous immunostimulatory molecule, chemotherapeutic drug, antibiotic, antifungal drug, antiviral drug, or combination thereof.

Another aspect of the present invention is to provide

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a therapeutic composition and a method of treating or preventing a disease in a mammal comprising administering to the mammal an effective amount of a recombinant virus and optionally an exogenous immunostimulatory or immunomodulator, chemotherapeutic drug antibiotic, antifungal drug, antiviral molecule, the amount effective in preventing or ameliorating the disease.

It is also an object of the present invention to provide a method of making an immune enhancing recombinant virus against a disease causing agent comprising inserting a nucleic acid sequence encoding one or more antigens or immunodominant epitopes thereof and a nucleic acid sequence encoding one or more immunostimulatory molecules into the genome or portion thereof of a recombinant virus.

It is also an object of the present invention to provide an immune enhancing recombinant virus against a disease causing agent comprising an immune enhancing recombinant virus comprising a nucleic acid sequence encoding one or more antigens or immunodominant epitopes thereof and a nucleic acid sequence encoding one or more immunostimulatory molecules.

It is also an object of the present invention to provide a vaccine against a disease causing agent comprising a recombinant virus containing a nucleic acid sequence encoding one or more antigens or immunodominant epitopes thereof and a nucleic acid sequence encoding one or more immunostimulatory molecules. The vaccine of the present invention is able to prevent or inhibit infection or disease caused by the disease causing agent.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other objects, feature and many of the attendant advantages of the invention will be better understood upon a reading of the detailed description of the invention.

Figure 1 shows β-Galactosidase production after BSC1 cell infection with various recombinant vaccinia virus

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constructs after 24 hr (■) or 36 hr (□) incubation.

Figure 2 shows the primary response of mice at Day 6 after injection I.V. with various recombinant vaccinia virus constructs as follows: GM-CSFrVV (□); IL-2rVV (●); TNFαrVV (▼); IFNγrVV(▽); VJS6 (O). Effector: Target cells ratios are plotted versus % specific ^{51}Cr release by targets.

Figure 3 shows the results from secondary cultures of mice at Day 6 after injection I.V. with various recombinant vaccinia virus constructs as follows: GM-CSFrVV (□); IL-2rVV (●); TNFαrVV (▼); IFNγrVV(▽); VJS6 (O). Effector: Target cells ratios are plotted versus % specific ^{51}Cr release by targets.

Figure 4 shows the results from secondary cultures of mice at Day 14 after injection I.V. with various recombinant vaccinia virus constructs as follows: GM-CSFrVV (□); IL-2rVV (●); TNFαrVV (▼); IFNγrVV(▽); VJS6 (O). Effector: Target cells ratios are plotted versus % specific ^{51}Cr release by targets.

Figure 5 shows the primary response of mice against vaccinia-infected CT26 tumor cells at Day 6 after injection S.C. with various recombinant vaccinia virus constructs as follows: GM-CSFrVV (●); IL-2rVV (□); TNFαrVV (▽); IFNγrVV(▼); VJS6 (■). Effector: Target cells ratios are plotted versus % specific ^{51}Cr release by targets.

Figure 6 shows the titration of lytic units 30% from a primary response of mice against vaccinia-infected CT26 tumor cells at Day 6 after injection S.C. with various recombinant vaccinia virus constructs as follows: GM-CSFrVV (●); IL-2rVV (□); TNFαrVV (▽); IFNγrVV(▼); VJS6 (■). Effector cells per well is plotted versus % specific ^{51}Cr release from CT26VAC target cells.

Figure 7 shows the results from secondary cultures of mice at Day 14 after injection S.C. with various recombinant vaccinia virus constructs as follows: GM-

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CSFrVV (□); IL-2rVV (●); TNFαrVV (▼); IFNγrVV(▽); VJS6
(O). Effector: target cells ratios are plotted versus %
specific ^{51}Cr release by targets.

Figure 8 shows active anti-tumor immunotherapy in
mice using exogenous IL-2 together with recombinant
vaccinia virus expressing a tumor associated antigen.
Treatment groups included HBSS (—); rVV+BG (— -);
HD IL-2 (100,000 I.U., I.P., BID x 3 days) (▪); rVV+BG +
HD IL-2 (100,000 I.U., I.P., BID x 3 days) (- - - -); rVV
+ BG + LD IL-2 (15,000 I.U., BID x 3 days (▪). Survival
time (days) is plotted versus proportion of surviving
mice.

Figure 9 shows survival data in an active-tumor
immunotherapy model in mice with an established non-
transduced tumor, CT26. GM-CSFrVV (▲); IL-2rVV (■);
TNFαrVV (▮); IFNγrVV(◆); VJS6 (▼); HBSS (●). Days after
tumor injection is plotted versus % surviving mice.

Figure 10 shows survival data in an active tumor
immunotherapy model in mice with established β-gal
expressing tumor, CT 26.C25. GM-CSFrVV (▲); IL-2rVV (■);
TNFαrVV (▮); IFNγrVV(◆); VJS6 (▼); HBSS (●). Days after
tumor injection is plotted versus % surviving mice.

Figure 11 shows active treatment of established
pulmonary metastases using various recombinant vaccinia
constructs. The average number of pulmonary metastases is
plotted versus recombinant vaccinia vector used for
treatment. HB2m = Human Beta-2-microglobulin rVV, Ld =
murine H-2L^d MCH Class I molecule rVV, NA = Neuramimidase
rVV, MVA = Ankara - attenuated vaccinia virus, Kb = murine
H-2K^b MCH Class I molecule rVV.

Figure 12 shows active treatment of established
pulmonary metastases using various recombinant vaccinia
constructs. The average number of pulmonary metastases is
plotted versus recombinant vaccinia vector used for
treatment. ICAM-1 = Intracellular adhesion molecule-1
rVV, D^d = murine H2-D^d MCH Class I molecule rVV, K^d =

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murine H-2 K^d MHC Class I molecule rVV, L^d = H-2 L^d MHC
Class I molecule rVV; K^b = H-2K^b MHC Class I molecule rVV;
HLA-A2.1 = Human MHC Class I molecule rVV, hβ_2m = Human
Beta-2-microglobulin rVV, NA = neuramimidase rVV, GM-CSF =
granulocyte, monocyte colony stimulatory factor rVV, IFNα
= interferon gamma rVV, TNFα = tumor necrosis factor alpha
rVV, MVA = Ankara - attenuated vaccinia virus, HBSS =
Hank's Balanced Salt Solution.

Figure 13 shows a Western blot analysis of
recombinant vaccinia virus expressed murine B7-1. BS-C-1
cells were infected with 1, v. MCB7-1; 2, v. MCMHA and
extracts were prepared 20 hours post infection. A murine
specific B7-1 hamster monoclonal antibody was used to
detect expression of recombinant proteins. ^{125}I protein A
(0.1 μCi/ml) and autoradiography were used to identify
bound antibody. Protein sizes were estimated using ^{14}C
molecular weight markers (MW).

Figure 14 shows the results of non-irradiated BALB/c
mice (5/group) which were injected intravenously with 5 x
10^5 tumor cells of CT26.WT or CT26.C25 on day #0. On day
#3 mice were immunized intravenously with different
recombinant vaccinia viruses (10^7 PFUs). Mice were
randomized, and euthanized on day #12. Lung were
harvested and stained, and pulmonary metastases were
enumerated in a blinded fashion. All mice injected with
CT26.WT had 500 metastases (data not shown). This graph
represents a compilation of data drawn from several
separate experiments. The individual rVV were involved in
the following number of experiments: v. IL2/β-gal -- five;
v. MCB7-1/β-gal -- six; v. D^d/β-gal -- one; v. K^d/β-gal --
one, v. L^d/β-gal -- one, v. K^b/β-gal -- one, v. HLA.A2.1/β-
gal -- one, v. mβ_2m/β-gal -- one, v. hβ_2m/β-gal -- one, v.
GM-CSF/β-gal -- four, v. IFN-γ/β-gal -- four, v. TNF-α/β-
gal -- four; v. JS6 -- greater than five, v. MVA/β-gal ---
one.

Figure 15 shows the results of non-irradiated BALB/c

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mice (10/group) which were injected intravenously with
HBSS, v. MCB7-1/β-gal, or v. MCMHA/β-gal. Twenty-one days
later mice were challenged intravenously with 5 x 10^5
tumor cells CT26.C25 or CT26.WT. Mice were randomized,
and euthanized on day #12. Lung were harvested and
stained, and pulmonary metastases were enumerated in a
blinded fashion. Duplicate experiments confirmed these
results.

Figure 16 shows the results of non-irradiated BALB/c
mice (10/group) which were injected with 10^5 or 10^7 PFUs
of v. MCB7-1/β-gal, v. MCMHA/β-gal, or HBSS on day #0.
Twenty-one days later a splenectomy was performed on all
immunized mice. 2 x 10^7 splenocytes from designated
groups of immunized mice were adoptively transferred to
similar mice (5/group) injected intravenously three days
earlier with 5 x 10^5 tumor cells of CT26.C25 or CT26.WT.
Designated groups of mice were treated with IL-2 (100,000
IU i.p. BID x 3 days). Mice were randomized and
euthanized nine days later. Lung were harvested and
stained, and pulmonary metastases were enumerated in a
blinded fashion. All mice inoculated with CT26.WT had
large tumor burdens (data not shown). Duplicate
experiments confirmed these results.

Figure 17 shows the results of non-irradiated BALB/c
mice (5/group) which were injected intravenously with 5 x
10^5 CT26.WT or CT26.C25 tumor cells on day #0. On day #3
mice were immunized intravenously with 10^7 PFUs of v.
MCB7-1/β-gal, v. MCMHA/β-gal, v. MCB7-1/NP or HBSS. On
day# 3 - 6, designated groups of mice were treated with
IL-2 (100,000 IU i.p. BID x 3 days). Mice were
randomized, and euthanized on day #12. Lung were
harvested and stained, and pulmonary metastases were
enumerated in a blinded fashion. All mice inoculated with
CT26.WT had large tumor burdens (data not shown). Four
separate experiments confirmed these results.

Figure 18 shows the results of non-irradiated BALB/c

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mice (5/group) which were injected intravenously with 5 x
10^5 CT26.WT or CT26.C25 tumor cells on day #0. On day #6
mice were immunized intravenously with 10^7 PFUs of v.
MCB7-1/β-gal, v. MCMHA/β-gal, v. MCB7-1/NP or HBSS. On
day# 6 - 9, designated groups of mice were treated with
IL-2 (100,000 IU i.p. BID x 3 days). Mice were
randomized, and euthanized on day #12. Lung were
harvested and stained, and pulmonary metastases were
enumerated in a blinded fashion. All mice inoculated
with CT26.WT had large tumor burdens (data not shown).
Duplicate experiments confirmed these results.

Figure 19 shows the results of non-irradiated
BALB/c mice (10/group) which were injected intravenously
with 5 x 10^5 CT26.WT or CT26.C25 tumor cells on day #0.
On day #3 mice were immunized intravenously with 10^7 PFUs
of v. MCB7-1/β-gal, v. MCMHA/β-gal, v. MCB7-1/NP or HBSS.
On day# 3 - 6, designated groups of mice were treated with
IL-2 (100,000 IU i.p. BID x 3 days). All mice
inoculated with CT26.WT were dead by day # 41 (data not
shown). Survival was followed daily and events recorded
as deaths.

Figure 20 shows the results of non-irradiated BALB/c
mice (5/group) which were injected intravenously with 5 x
10^5 CT26.WT or CT26.C25 tumor cells on day #0. On day #3
mice were immunized intravenously with 10^7 PFUs of v.
MCB7-1/β-gal, v. MCB7-2/β-gal, v. MCB7-1/B7-2/β-gal, v.
MCMHA/B7-2/β-gal, v. MCMHA/β-gal, v. MCB7-1/NP, or HBSS.
On day# 3 - 6, designated groups of mice were treated with
IL-2 (100,000 IU i.p. BID x 3 days). Mice were
randomized, and euthanized on day #12. Lung were
harvested and stained, and pulmonary metastases were
enumerated in a blinded fashion. All mice inoculated with
CT26.WT had large tumor burdens (data not shown).
Duplicate experiments confirmed these results.

Figures 21a through 21f show a FACS analysis of
splenocytes isolated from normal non-depleted (Figs. 21a

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and 21b), T$_{CD8+}$-depleted (Figs. 21c and 21d), or T$_{CD4+}$-depleted (Figs. 21e and 21f) mice. Mice were given two i.v. injections of GK1.5 at 100 mg/ml or of empirically determined levels of 2.43 monoclonal antibodies 48 hours prior to receiving tumor challenge, and again 6 days later. Upper left hand panel in each square displays percent CD8+ cells, upper right panel shows percent CD4+CD8+ cells, lower right shows percent CD4+, and lower left shows scatter for all cells. Figure 21a, no depletion Using fluorescein isothiocyante-labeled anti-CD4 and phycoerythrin labeled anti-CD8 antibodies, FACS analysis was performed 1 day prior to immunization, and again at day 7 to verify depletion.

Figure 21g shows the mean number of pulmonary metastases of CD4- and CD8- immuno depleted mice vaccinated with V.B7-1/β-gal. Prior to tumor challenge non-irradiated BALB/c mice (5/group) were injected with anti-CD4 and anti-CD8 monoclonal antibodies (GKI.5 (anti-CD4) and 2.43 (anti-CD8). On day#0 mice were injected intravenously with 5 x 10^5 CT26.C25 tumor cells. On day #3 mice were immunized intravenously with 10^7 PFUs of v. MCB7-1/β-gal, v. MCMHA/β-gal, v. MCB7-1/NP or HBSS. On day #6, the mice were again injected with anti-CD4 and anti-CD8 monoclonal antibodies. Mice were randomized, and euthanized on day #12. Lung were harvested and stained, and pulmonary metastases were enumerated in a blinded fashion. FACS analysis was performed 1 day prior to immunization, and again at day 7 to verify depletion. Only data from v.B7-1/B-gal vaccination groups is shown. Duplicate experiments confirmed these results.

Figures 22a and 22b show active immunotherapy is enhanced when exogenous rIL-2 and rVV are given in concert. BALB/c mice (five per group) were challenged i.v. with 5 x 10^5 CT26.WT (Fig. 22a) or CT26.CL25 tumor cells (Fig. 22b). After 3 days they received a single i.v. injection of medium alone (HBSS) or medium containing

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5 x 10^6 PFU of different TK⁻ rVV either expressing (VJS6) or not expressing (V69) β-gal. Two different regimens of rIL-2 administration were started 12 h after rVV injection: high dose (HD, 100,000 Cetus U, twice a day, i.p. for 3 days) or low dose (LD, 15,000 Cetus U, twice a day, i.p. for 5 days). Mice were checked twice a day for survival.

Figures 23a and 23b show that exogenous rIL-2 enhances the function of rFPV. BALB/c mice (five per group) were inoculated i.v. with 5 x 10^5 CT26.WT (Fig. 23a) or CT26.CL25 tumor cells (Fig. 23b). On day 3 after tumor injection, they received a single i.v. injection of the following viruses: no virus (HBSS alone), 10^7 PFU of FPV.bg40k (rVPV), or FPV wild-type (FPVwt). rIL-2 (100,000 Cetus U, twice a day) was administered i.p. starting 12 h after FPV injection and continued for 3 days. Mice were checked twice a day for survival.

Figure 24 shows exogenous rIL-2 plus rVV is therapeutic in the more advanced 6-day tumor model. BALB/c, mice (five per group) were inoculated i.v., with 10^{5}CT26.CL25 tumor cells. Six days after tumor injection, they received the same treatments described in Figures 22a and 22b with the exception that only the highest dose of rIL-2 was administered. Mice were checked daily for survival. No prolongation of survival was obtained by the various treatments in mice bearing 6-day-old pulmonary metastases of CT26.WT tumor (data not shown).

Figures 25a and 25b show a drVV expressing IL-2, but not GM-CSF, IFN-γ, or TNF-α, significantly reduces the number of pulmonary metastases in a 3-day model. Five BALB/c mice per group were injected i.v. with 5 x 10^5 tumor cells of either CT26.WT or CT26.CL25 cell lines. Three days later they received a single i.v. injection of HBSS alone (none) or containing 5 x 10^{6}PFU/mouse of different rVV, as indicated. On day 12 post tumor challenge, lungs were harvested and pulmonary nodules were

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enumerated in a blind fashion. An independent repeat of
this experiment gave identical results.

Figures 26a and 26b show the function of a drVV
expressing IL-2 is further enhanced when additional
exogenous IL-2 is provided. BALB/c mice (five per group)
were challenged i.v. with 5×10^5 CT26.WT (Fig. 26a) or
CT26.CL25 tumor cells (Fig. 26b). After 3 days they
received a single i.v. injection of plain medium (HBSS) or
medium containing 5×10^6 PFU of rVV encoding β-gal alone
(VJS6) or together with IL-2 (IL-2 rVV). Twelve hours
after rVV, 100,000 rIL-2U were inoculated, according to
the regimen described in Figs. 22a and 22b. Mice were
checked twice a day for survival.

Figure 27 shows that expression of IL-2 by drVV
enhances the antivaccinia CTL response. Two BALB/c mice
were immunized with 5×10^6 PFU/mouse of different rVV.
After 6 days the spleens were aseptically removed, mixed
together, and tested directly in a 6-h ^{51}Cr release assay
against CT26.WT tumor cell line, either infected (CT26
vaccinia) or noninfected (CT26.WT) during the isotope
labeling with more than 10:1 moi of crude 19 VV
preparation. Spontaneous release of target cells never
exceeded 20%. E:T cell ratio was 100:1 and then 1:3
dilutions. Lytic units 30% (L.U. 30%) indicate the number
of effector cells required to obtain 30% lysis of 10,000
target cells. L.U. 30% were normalized for the total
number of cells recovered for each spleen and expressed as
total L.U./spleen.

Figure 28 shows that the presence of tumor cells
specifically enhance the CTL response elicited by IL-2 rVV
in a dose-dependent manner. BALB/c mice were injected
with HBSS alone or with varying doses of CT26.WT or
CT26.CT25 as specified. After 3 days, mice were immunized
with 5×10^6 PFU/mouse of either VJS6 or IL-2 rVV. On day
9 after tumor challenge (day 6 after vaccination) the
primary cytotoxic response was evaluated in a 6-h ^{51}Cr

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release assay against CT26.WT, CT26.WT pulsed with the β-gal L^d-restricted peptide (CT26T + peptide), CT26.CL25, and the irrelevant target cells E22 as shown. The effects of escalating doses of tumor on the generation of the primary cytotoxic response is shown for the immunization with IL-2rVV in nontumor-bearing mice (▲), or in mice bearing 5 x 10^4 CT26.CL25 (▲), 1 x 10^5 CT26.CL25 (●), 5 x 10^5 CT26.CL25 (O), or the highest dose, 5 x 10^5 of the parental (non-β-gal-expressing) CT26.WT cell line (□). An additional control is the non-IL-2-expressing VJS6 virus injected in mice bearing 5 x 10^5 CT26.CL25 (■).

Figure 29 shows the effects of recombinant IL-10 on the therapeutic effectiveness of immunization with the vaccinia virus, VJS6 + IL-1 (●); VJS6 alone (X).

Figure 30 shows the IL-10 adjuvant therapy on the therapeutic effectiveness of immunization with various concentrations of recombinant vaccinia virus encoding the model tumor antigen.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is a novel recombinant virus expressing an antigen(s) from a disease causing agent and an immunostimulatory molecule(s). The novel recombinant virus is capable of eliciting or upregulating an immune response in a mammal to T-dependent antigens for the purpose of preventing or treating a disease. The novel recombinant virus of the present invention is particularly important in upregulating cell-mediated immunity.

Cell-mediated immunity is crucial to resistant to diseases caused by cancer and pathogenic microorganism, particularly viruses and other intracellular microorganisms.

The recombinant virus has incorporated into its genome or portion thereof a gene encoding an antigen from a disease causing agent and one or more genes encoding one or more immunostimulatory molecules. A host cell infected

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with the recombinant virus expresses both the antigen(s)
from a disease causing agent and expresses the
immunostimulatory molecule(s). The antigen may be
expressed at the cell surface of the infected host cell.
The immunostimulatory molecule may be expressed at the
cell surface or may be actively secreted by the host cell.

The expression of both the antigen and the
immunostimulatory molecule provides the necessary MHC
restricted peptide to specific T cells and the appropriate
signal to the T cell to aid in antigen recognition and
proliferation or clonal expansion of antigen specific T
cells. The overall result is an upregulation of the
immune system. In a preferred embodiment the upregulation
of the immune response is an increase in antigen specific
cytotoxic lymphocytes which are able to kill or inhibit
the growth of a disease causing agent or a cell infected
with a disease causing agent.

In one embodiment, the recombinant virus comprises
the virus genome or portions thereof, the nucleic acid
sequence encoding an antigen from a pathogenic
microorganism and one or more nucleic acid sequences
encoding one or more immunostimulatory molecules.

In another embodiment, the recombinant virus
comprises the virus genome or portions thereof, the
nucleic acid sequence encoding a tumor associated antigen,
and one or more nucleic acid sequences encoding one or
more immunostimulatory molecules.

In one embodiment the recombinant viruses have been
constructed and co-express model tumor antigens together
with cytokines (TNF-α, IFN-γ, GM-CSF, IL-10 and IL-2),
restriction elements (class 1 α-chains and β_2m), and co-
stimulatory and accessory molecules (B7-1, B7-2 and ICAM-1
and the like) alone and in a variety of combinations.
Simultaneous production of an immunostimulatory molecule
and the model TAA at the site of virus
replication/infection (in any case, the site of TAA

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production) enhances the generation of specific effectors.
Dependent upon the specific immunostimulatory molecules,
different mechanisms might be responsible for the enhanced
immunogenicity: augmentation of help signal (IL-2),
recruitment of professional APC (GM-CSF), increase in CTL
frequency (IL-2), effect on antigen processing pathway and
MHC expression (IFNγ and TNFα) and the like. The co-
expression of a model antigen together with at least one
immunostimulatory molecule is effective in an active
immunotherapy model.

The present invention also encompasses a recombinant
virus comprising the virus genome or portion thereof, the
nucleic acid sequence encoding the antigen of interest and
more than one nucleic acid sequences encoding more than
one immunostimulatory molecule for the added benefit of
upregulating an immune response against the antigen.

In some cases it may be beneficial to make a
recombinant virus comprising more than one antigen of
interest for the purpose of having a multivalent vaccine.
For example, the recombinant virus may comprise the virus
genome or portions thereof, the nucleic acid sequence
encoding GP120 (from HIV), the nucleic acid sequence
encoding Hep B surface antigen and one or more
immunostimulatory molecules.

In one embodiment, the recombinant virus comprises
the vaccinia virus genome or portions thereof, the nucleic
acid sequence encoding MART-1 and the nucleic acid
sequence encoding the immunostimulatory molecule, B 7.1
alone or in combination with the nucleic acid sequence
encoding the immunostimulatory molecule, B7.2.

In another embodiment, the recombinant virus
comprises the fowlpox virus genome or portions thereof,
the nucleic acid sequence encoding MART-1, the nucleic
acid sequence encoding MAGE-1, and the nucleic acid
sequence encoding the immunostimulatory molecule, IL-2,
alone or in combination with the nucleic acid sequence

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encoding the immunostimulatory molecule, B7.1. In a
specific embodiment the recombinant virus is McB7-1,
McB71-1/NP, McB7-1β-gal, McB7-1/ova, McB7-1/PIA, McB7-
1/B7-2, McMHA, McMHA/NP, McMHA/β-gal, McMHA/ova,
McMHA/PIA, McMHA/B7-2, McB7-2, McB7-2/β-gal, IL-2(β-
gal)rVV, GM-CSF(β-gal)rVV, IFNγ(β-gal)rVV, IL-10(β-gal)
rVV, and TNFα(β-gal)rVV.

The insertion of costimulatory molecules and/or
cytokine genes in recombinant vaccinia virus (rVV genome
containing the model TAA) is beneficial in treatment of
established metastases.

The present invention encompassed a composition
comprising a recombinant virus containing a viral genome
or portion thereof, one or more nucleic acid sequences
encoding one or more antigens from one or more disease
causing agents and one or more nucleic acid sequences
encoding one or more immunostimulatory molecules. The
compositions of the present invention may also comprise an
exogenous immunostimulatory molecule or combinations of
immunostimulatory molecules and/or may comprise a
chemotherpeutic drug, antibiotic, antifungal drug,
antiviral drug and the like and combinations thereof.

In one embodiment, the composition contains a
recombinant virus which has incorporated into its genome
or portion thereof a gene encoding a tumor associated
antigen and exogenous IL-2. In another embodiment, the
composition contains a recombinant virus which as
incorporated into its genome or portion thereof a gene
encoding a tumor associated antigen in combination with
exogenous IL-10.

Virus Vectors

Virus that may be used in the present invention are
those in which a portion of the genome can be deleted to
introduce new genes without destroying infectivity of the
virus. The virus vector of the present invention is a
nonpathogenic virus. In one embodiment the virus vector

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has a tropism for a specific cell type in the mammal. In
another embodiment, the virus vector of the present
invention is able to infect professional antigen
presenting cells such as dendritic cells and macrophages.
In yet another embodiment of the present invention, the
virus vector is able to infect any cell in the mammal.
The virus vector may also infect tumor cells.

The virus of the present invention include but is not
limited to Poxvirus such as vaccinia virus, fowlpox virus
and a highly attenuated vaccinia virus (Ankara or (MVA)),
retrovirus, adenovirus, baculovirus and the like.

The vaccinia virus genome is known in the art. It is
composed of a HIND F13L region, TK region, and an HA
region. The recombinant vaccinia virus has been used in
the art to incorporate an exogenous gene for expression of
the exogenous gene product (Perkus et al. Science 229:981-
984, 1985; Kaufman et al. Int. J. Cancer 48:900-907, 1991;
Moss Science 252:1662, 1991).

A general strategy for construction of vaccinia virus
expression vectors have been described (Smith and Moss Bio
Techniques Nov/Dec, p. 306-312, 1984; U.S. Patent No.
4,738,846). Briefly, the first step in formation of
recombinant viruses expressing heterologous DNA is the
construction of a chimeric gene containing a vaccinia
promoter fused to the protein coding sequences of the
foreign gene. The chimeric gene is assembled in a plasmid
vector and engineered so that the transcriptional start
site of the promoter is positioned close to the
translational initiation codon of the foreign gene.
Importantly, additional ATG triplets between the
transcriptional and translational start sites should be
eliminated so that fusion polypeptides or incorrect
reading frames are avoided. Generally, the promoters are
included in DNA fragments 200-300 base pairs in length,
but smaller fragments have also proved functional. After
their assembly in plasmid vectors, the chimeric genes are

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inserted into the virus genome by homologous recombination
in vivo. This method is necessary since the great size of
vaccinia DNA makes *in vitro* construction of recombinant
molecules impractical. To facilitate homologous
recombination, the chimeric gene is first flanked by
vaccinia DNA taken from a non-essential region of the
virus genome. The resulting plasmid, called a
recombination vector, is transfected into vaccinia virus-
infected cells, whereupon homologous recombination results
in site-specific insertion of the chimeric gene into the
virus genome. Recombinant genomes are replicated and
packaged into infectious progeny virus within the infected
cells. The nature of the flanking DNA is important since
this determines the site of insertion. Only nonessential
regions can be used and several of these have now been
identified. A commonly used locus is the vaccinia TK gene
since recombinant viruses are consequently TK.

          A gene encoding an antigen of a disease causing agent
may be incorporated into the HIND F13L region or
alternatively incorporated into the TK region of
recombinant vaccinia virus vector. Likewise, a gene
encoding an immunostimulatory molecule may be incorporated
into the HIND F13L region or the TK region of recombinant
vaccinia virus vector.

          Sutter and Moss (*Proc. Nat'l. Acad. Sci U.S.A.*
89:10847-10851, 1992) and Sutter et al. (*Virology* 1994)
disclose the construction and use as a vector, the non-
replicating recombinant Ankara virus (MVA, modified
vaccinia Ankara) which may be used as a viral vector in
the present invention.

          Baxby and Paoletti (*Vaccine* 10:8-9, 1992) disclose
the construction and use as a vector, of the non-
replicating poxvirus, including canarypox virus, fowlpox
virus and other avian species which may be used as a viral
vector in the present invention.

          Expression vectors suitable for use in the present

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invention comprise at least one expression control element operationally linked to the nucleic acid sequence. The expression control elements are inserted in the vector to control and regulate the expression of the nucleic acid sequence. Examples of expression control elements includes, but is not limited to, lac system, operator and promoter regions of phage lambda, yeast promoters and promoters derived from polyoma, adenovirus, retrovirus or SV40. Additional preferred or required operational elements include, but are not limited to, leader sequence, termination codons, polyadenylation signals and any other sequences necessary or preferred for the appropriate transcription and subsequent translation of the nucleic acid sequence in the host system. It will be understood by one skilled in the art the correct combination of required or preferred expression control elements will depend on the host system chosen. It will further be understood that the expression vector should contain additional elements necessary for the transfer and subsequent replication of the expression vector containing the nucleic acid sequence in the host system. Examples of such elements include, but are not limited to, origins of replication and selectable markers. It will further be understood by one skilled in the art that such vectors are easily constructed using conventional methods (Ausubel et al., (1987) in "Current Protocols in Molecular Biology", John Wiley and Sons, New York, New York) or commercially available.

Disease Causing Agents

The recombinant virus of the present invention is effective in treating or preventing disease caused by disease causing agents or a disease state. Each disease causing agent or disease state has associated with it an antigen or immunodominant epitope on the antigen which is crucial in immune recognition and ultimate elimination or control of the disease causing agent in a mammal,

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sometimes referred to in the art as a protective antigen.
The mammalian immune system must come in contact with the
antigen or immunodominant epitope on the antigen in order
to mount a humoral and/or cellular immune response against
the associated disease causing agent.

The recombinant virus of the present invention
comprises the one or more nucleic acid sequences encoding
one or more isolated antigens or immunodominant epitopes
on the antigens and one or more nucleic acid sequences
encoding one or more immunostimulatory molecules for the
purpose of enhancing immune response against the disease
causing agent.

Such disease causing agents include but are not
limited to cancer and pathogenic microorganisms or
mammals. Mammals include but are not limited to humans,
primates, rats, mice, guinea pigs, rabbits, horses, cows,
sheep, pigs, goats and the like. Cancers of mammals which
may be treated using the recombinant virus of the present
invention include but are not limited to melanoma,
metastases, adenocarcinoma, thymoma, lymphoma, sarcoma,
lung cancer, liver cancer, colon cancer, non-Hodgkins
lymphoma, Hodgkins lymphoma, leukemias, uterine cancer,
breast cancer, prostate cancer, ovarian cancer, cervical
cancer, bladder cancer, kidney cancer, pancreatic cancer
and the like.

The term melanoma includes, but is not limited to,
melanomas, metastatic melanomas, melanomas derived from
either melanocytes or melanocytes related nevus cells,
melanocarcinomas, melanoepitheliomas, melanosarcomas,
melanoma in situ, superficial spreading melanoma, nodular
melanoma, lentigo maligna melanoma, acral lentiginous
melanoma, invasive melanoma or familial atypical mole and
melanoma (FAM-M) syndrome. Such melanomas in mammals may
be caused by, chromosomal abnormalities, degenerative
growth and developmental disorders, mitogenic agents,
ultraviolet radiation (UV), viral infections,

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inappropriate tissue expression of a gene, alterations in
expression of a gene, and presentation on a cell, or
carcinogenic agents.

The aforementioned cancers can be assessed or treated
by methods described in the present application. In the
case of cancer, a gene encoding an antigen associated with
the cancer is incorporated into the recombinant virus
genome or portion thereof along with a gene encoding one
or more immunostimulatory molecules. The antigen
associated with the cancer may be expressed on the surface
of a cancer cell or may be an internal antigen. In one
embodiment the antigen associated with the cancer is a
tumor associated antigen (TAA) or portion thereof.
Examples of TAA that may be used in the present invention
include but are not limited to melanoma TAAs which include
but are not limited to MART-1 (Kawakami et al. J. Exp.
Med. 180:347-352, 1994), MAGE-1, MAGE-3, GP-100, (Kawakami
et al. Proc. Nat'l. Acad. Sci. U.S.A. 91:6458-6462, 1994),
CEA, TRP-1, P-15, and tyrosinase (Brichard et al. J. Exp.
Med. 178:489, 1993) and the like.

The nucleotide sequence of the MAGE-3 gene is
disclosed in Gaugler et al. J. Exp. Med. 179:921-930,
1994. MAGE-3 is expressed on many tumors of several
types, such as melanoma, head and neck squamous cell
carcinomas, lung carcinoma and breast carcinoma but not in
normal tissues except for testes. The approximately 1.6
Kilobase (kb) cDNA of MART-1 was cloned into a vector and
the resulting plasmid, deposited with the American Type
Culture Collection (ATCC) 12301 Parklawn Drive, Rockville,
MD 20852 USA on April 14, 1994, and given ATCC Deposit
Number 75738. The cloning of MART-1 is disclosed in
KawaKami et al J. Exp. Med. 180:347-352, 1994 and Serial
No. 08/231,565 filed April 22, 1994. The full length
MART-1 nucleic acid sequence can be isolated from the
pCRII plasmid by digestion with HINDII and XhoI
restriction enzymes. This 1.6kb nucleic acid sequence or

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portions thereof can then be incorporated into the genome
of the recombinant viruses described herein along with an
immunostimulatory gene or genes.

In another embodiment the TAAs are CA-19-A
(pancreatic cancer), CA-125 (ovarian cancer), PSA
(prostate cancer), erb-2 (breast cancer, CA-171A) and the
like (Boon et al. Ann. Rev. Immunol 12:337, 1994).

The present invention is in no way limited to the
genes encoding the above listed TAAs. Other TAAs may be
identified, isolated and cloned by methods known in the
art such as those disclosed in U.S. Patent No. 4,514,506.

Genes encoding an antigen of a disease causing agent
in which the agent is a pathogenic microorganism in
mammals and include viruses such as HIV (GP-120, p17, GP-
160 antigens), influenza (NP, HA antigen), herpes simplex
(HSVdD antigen), human papilloma virus, equine
encephalitis virus, hepatitis (Hep B Surface Antigen)
feline leukemia virus, canine distemper, rabies virus, and
the like. Pathogenic bacteria include but are not limited
to Chlamydia, Mycobacteria, Legioniella and the like.
Pathogenic protozoans include but are not limited to
malaria, Babesia, Schistosoma, Toxiplasma, Toxocara canis,
and the like. Pathogenic yeast include Aspergillus,
invasive Candida, and the like. In a preferred embodiment
the pathogenic microorganism is an intracellular organism.

Immunostimulatory Molecules: Costimulation/Accessory
Molecules and Cytokines

The gene from costimulation/accessory molecule and/or
gene encoding an a cytokine in combination with a gene
encoding an antigen from a disease causing agent is
incorporated into the genome of a recombinant virus.
Examples of costimulation molecules include but are not
limited to B7-1, B7-2, ICAM-1, ICAM-2, LFA-1, LFA-3, CD72
and the like. Examples of cytokines encompassed by the
present invention include but are not limited to IL-2, IL-

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1, IL-3 through IL-9, IL-11, IL-13 through IL-15, G-CSF, M-CSF, GM-CSF, TNFα, IFNα, IFNγ, IL-10, IL-12, Regulated upon activation, normal T expressed and presumably secrected cytokine (RANTES), and the like. Examples of chemokines encompassed by the present invention include but are not limited to CTAP III, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MBSA, MIP-1α, MIP-1B and the like.

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IL-2 construct

The IL-2 gene of the present invention was made made as disclosed by Taniguchi et al (Structure and expression of a cloned cDNA for human interleukin-2 Nature 302:305, 1983).

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In one embodiment the entire IL-2 gene as disclosed in Taniguchi et al is incorporated into the TK gene sequence of vaccinia virus.

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The promotor sequence for the IL-2 construct of the present invention is made up of the P synthetic late promotor as disclosed in Davidson et al Nucleic Acid Research 18 (No. 14):4285-4286, 1991.

Also encompassed in the present invention is a chimeric gene containing a pox virus promotor region linked to the coding segment of one or more foreign genes encoding an antigen(s) from a disease causing agent and the coding segment of one or more foreign genes encoding an immunostimulatory molecule(s). The chimeric genes then incorporated into the pox virus genome by homologous recombination in cells that have transfected with a plasmid vector containing the chimeric gene and infected with the pox virus.

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In a one embodiment the IL-2 construct of the present invention comprises the recombinant vaccinia containing the IL-2 gene and the P synthetic late promotor and an antigen in the TK region from a disease causing agent in the F13L region of the vaccinia virus genome promotor.

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B7 Construct

Co-stimulatory molecules of the B7 family (namely

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B7.1, B7.2, and possibly B7.3) represent a more recently
discovered, but important group of molecules. B7.1 and
B7.2 are both member of the Ig gene superfamily. These
molecules are present on macrophages, dendritic cells,
monocytes, i.e., antigen presenting cells (APCs). If a
lymphocyte encounters an antigen alone, with co-
stimulation by B7.1, it will respond with either anergy,
or apoptosis (programmed cell death); if the co-
stimulatory signal is provided it will respond with clonal
expansion against the target antigen. No significant
amplification of the immune response against a given
antigen occurs without co-stimulation (June et al.
(Immunology Today 15:321-331, 1994); Chen et al.
(Immunology Today 14:483-486); Townsend et al. (Science
259:368-370)). Freeman et al. (J. Immunol. 143:2714-2722,
1989) report cloning and sequencing of B7.1 gene. Azuma
et al. (Nature 366:76-79, 1993) report cloning and
sequencing B7.2 gene.

In one embodiment the B7.1 gene was inserted into the
Hind F13L region of the vaccinia virus, with the β-gal
placed in the TK region. The construct for B7.2 and
B7.1/B7.2 in conjunction with a tumor antigen are prepared
in the same fashion as B7.1.

In another embodiment the B7 gene is inserted into
the TK region of vaccinia virus and the gene encoding β-
gal inserted in the Hind F13L region of the vaccinia
virus.

The IFNγ construct, TNFα construct, GM-CSF construct
and ICAM-1 construct were constructed as disclosed in
Davidson et al Nucleic Acid Research 18 (No. 14):4285-
4286, 1991.

The present invention also encompasses methods of
treatment or prevention of a disease caused by the disease
causing agents disclosed here.

In the method of treatment, the administration of the
recombinant virus of the invention may be for either

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"prophylactic" or "therapeutic" purpose. When provided
prophylactically, the recombinant virus of the present
invention is provided in advance of any symptom. The
prophylactic administration of the recombinant virus
serves to prevent or ameliorate any subsequent infection
or disease. When provided therapeutically, the
recombinant virus is provided at (or shortly after) the
onset of a symptom of infection or disease. Thus the
present invention may be provided either prior to the
anticipated exposure to a disease causing agent or after
the initiation of the infection or disease.

The genetic definition of tumor-specific antigens
allows for the development of targeted antigen-specific
vaccines for cancer therapy. Insertion of a tumor antigen
gene in the genome of viruses in combination with a
immunostimulatory molecule is a powerful system to elicit
a specific immune response in terms of prevention in
patient with an increased risk of cancer development
(preventive immunization), prevention of disease
recurrence after primary surgery (anti-metastatic
vaccination), or as a tool to expand the number of CTL in
vivo, thus improving their effectiveness in eradication of
diffuse tumors (treatment of established disease).
Finally, recombinant viruses of the present invention can
elicit an immune response in patient that is enhanced ex
vivo prior to being transferred back to the tumor bearer
(adoptive immunotherapy).

The term "unit dose" as it pertains to the inoculum
refers to physically discrete units suitable as unitary
dosages for mammals, each unit containing a predetermined
a quantity of recombinant virus calculated to produce the
desired immunogenic effect in association with the
required diluent. The specifications for the novel unit
dose of an inoculum of this invention are dictated by and
are dependent upon the unique characteristics of the
recombinant virus and the particular immunologic effect to

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be achieved.

The inoculum is typically prepared as a solution in tolerable (acceptable) diluent such as saline, phosphate-buffered saline or other physiologically tolerable diluent and the like to form an aqueous pharmaceutical composition.

The route of inoculation may be intravenous (I.V.), intramuscular (I.M.), subcutaneous (S.C.), intradermal (I.D.) and the like, which results in eliciting a protective response against the disease causing agent. The dose is administered at least once. Subsequent doses may be administered as indicated.

In providing a mammal with the recombinant virus of the present invention, preferably a human, the dosage of administered recombinant virus will vary depending upon such factors as the mammal's age, weight, height, sex, general medical condition, previous medical history, disease progression, tumor burden and the like.

In general, it is desirable to provide the recipient with a dosage of recombinant virus in the range of from about 10^5 to about 10^{10} plaque forming units/mg mammal, although a lower or higher dose may be administered.

The recombinant viral vector can be introduced into a mammal either prior to any evidence of cancers such as melanoma or to mediate regression of the disease in a mammal afflicted with a cancer such as melanoma. Examples of methods for administering the viral vector into mammals include, but are not limited to, exposure of cells to the recombinant virus ex vivo, or injection of the recombinant virus into the affected tissue or intravenous S.C., I.D. or I.M. administration of the virus. Alternatively the recombinant viral vector or combination of recombinant viral vectors may be administered locally by direct injection into the cancerous lesion or topical application in a pharmaceutically acceptable carrier. The quantity of recombinant viral vector, carrying the nucleic acid

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sequence of one or more TAAs to be administered is based
on the titer of virus particles. A preferred range of the
immunogen to be administered is 10^5 to 10^{10} virus particles
per mammal, preferably a human.

After immunization the efficacy of the vaccine can be
assessed by production of antibodies or immune cells that
recognize the antigen, as assessed by specific lytic
activity or specific cytokine production or by tumor
regression. One skilled in the art would know the
conventional methods to assess the aforementioned
parameters. If the mammal to be immunized is already
afflicted with cancer or metastatic cancer the vaccine can
be administered in conjunction with other therapeutic
treatments.

In one method of treatment, autologous cytotoxic
lymphocytes or tumor infiltrating lymphocytes may be
removed from the patient with cancer as disclosed in U.S.
Patent No. 5,126,132 and U.S. Patent No. 4,690,915. The
lymphocytes are grown in culture and antigen specific
lymphocytes expanded by culturing in the presence of the
recombinant virus of the present invention. The antigen
specific lymphocytes are then reinfused back into the
patient.

The present invention also encompasses combination
therapy. By combination therapy is meant that the
recombinant virus containing one or more genes encoding
one or more antigens associated with one or more disease
agents and one or more genes encoding one or more
immunostimulatory molecules is administered to the patient
in combination with other exogenous immunomodulators or
immunostimulatory molecules, chemotherapeutic drugs,
antibiotics, antifungal drugs, antiviral drugs and the
like alone or in combination thereof. In one embodiment
the combination therapy includes a recombinant virus and
exogenous IL2. In another embodiment the combination
therapy includes a recombinant virus encoding one or more

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exogenous antigens and exogenous IL-10. Examples of other
exogenously added agents include exogenous IL-2, IL-6, IL-
12, GM-CSF, interferon, IL-10, tumor necrosis factor,
RANTES (Promega, G5661), cyclophosphamide, and cisplatin,
gancyclovir, amphotericin B and the like.

This invention further comprises an antibody or
antibodies elicited by immunization with the recombinant
virus of the present invention. The antibody has
specificity for and reacts or binds with the antigen of
interest. In this embodiment of the invention the
antibodies are monoclonal or polyclonal in origin.

Exemplary antibody molecules are intact
immunoglobulin molecules, substantially intact
immunoglobulin molecules or those portions of an
immunoglobulin molecule that contain the antigen binding
site, including those portions of immunoglobulin molecules
known in the art as F(ab), F(ab'); F(ab')$_2$ and F(v).
Polyclonal or monoclonal antibodies may be produced by
methods known in the art. (Kohler and Milstein (1975)
Nature 256, 495-497; Campbell "Monoclonal Antibody
Technology, the Production and Characterization of Rodent
and Human Hybridomas" in Burdon et al. (eds.) (1985)
"Laboratory Techniques in Biochemistry and Molecular
Biology," Volume 13, Elsevier Science Publishers,
Amsterdam). The antibodies or antigen binding fragments
may also be produced by genetic engineering. The
technology for expression of both heavy and light chain
genes in E. coli is the subject of the PCT patent
applications: publication number WO 901443, WO 901443 and
WO 9014424 and in Huse et al. (1989) Science 246:1275-
1281.

In one embodiment the antibodies of this invention
are used in immunoassays to detect the novel antigen of
interest in biological samples.

In one embodiment, the MART-1 antibodies of this
invention generated by immunization with recombinant

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vaccinia virus expressing MART-1 and B7.1 are used to assess the presence of the MART-1 antigen from a tissue biopsy of a mammal afflicted with melanoma using immunocytochemistry. Such assessment of the delineation of the MART-1 antigen in a diseased tissue can be used to prognose the progression of the disease in a mammal afflicted with the disease or the efficacy of immunotherapy. Conventional methods for immunohistochemistry are described in (Harlow and Lane (eds) (1988) In "Antibodies A Laboratory Manual", Cold Spinning Harbor Press, Cold Spring Harbor, New York; Ausbel et al. (eds) (1987). In Current Protocols In Molecular Biology, John Wiley and Sons (New York, New York).

In another embodiment the antibodies of the present invention are used for immunotherapy. The antibodies of the present invention may be used in adoptive immunotherapy.

In providing a patient with the antibodies or antigen binding fragments to a recipient mammal, preferably a human, the dosage of administered antibodies or antigen binding fragments will vary depending upon such factors as the mammal's age, weight, height, sex, general medical condition, previous medical condition and the like.

In general, it is desirable to provide the recipient with a dosage of antibodies or antigen-binding fragments which is in the range of from about 1 mg/Kg to about 10 mg/Kg body weight of the mammal, although a lower or higher dose may be administered.

The antibodies or antigen-binding fragments of the present invention are intended to be provided to the recipient subject in an amount sufficient to prevent, lessen or attenuate the severity, extent or duration of the disease or infection.

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Example 1
Construction and Characterization of Recombinant Vaccinia Viruses Expressing Murine B7 (mB7) and Additional Foreign Proteins

Various methods for inserting foreign genes into a
5   vaccinia virus expression vector are known in the art
(Smith, G.L. et al, Vaccinia Virus Expression Vectors:
Construction, Properties and Applications Bio Techniques
Nov/Dec:306-311, 1984; Flexner, C. et al. Expression of
Human Interleukin-1 by live recombinant vaccinia virus.
10 Vaccines 87, Cold Spring, Harbor Lab., pp 380-383; U.S.
Patent No. 4,738,846). These as well as other techniques
may be employed to construct the recombinant virus of the
present invention. Recombinant vaccinia viruses
expressing mB7 and additional extrinsic proteins were
15 constructed. The mB7 and additional foreign genes were
inserted (by homologous recombination) into the vaccinia
VP37 (Hind III F) and TK (Hind III J) genes. For control
viruses, the mB7 gene was replaced by the measles HA (mHA)
gene.
20 Construction and Characterization of Vaccinia Recombinant
Viruses Expressing mB7-1, mB7-2 and mHA.

The construction of initial recombinants expressing
the above genes employed the plaque formation selection
system in which the foreign gene is directed into the
25 vaccinia F13L (VP37) loci. Briefly, the foreign gene is
cloned into a vaccinia transfer plasmid (pRB21) adjacent
to the synthetic early late promoter (E/L) and flanked by
DNA homologous to the F13L region in vaccinia. A full-
length F13L gene (under control of its authentic promoter)
30 is also situated within the flanking regions. The mB7-1,
Mb7-2 and mHA were individually cloned adjacent to E/L
within pRB21. A virus that has a plaque deficient
phenotype (due to deletion of the F13L plaque forming
gene) was used for transfection and the proceeding
35 homologous recombination of plasmid DNA. Transfections

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were carried out according to standard published
procedures. Recombinant viruses were readily identified
according to their plaque positive phenotype, in contrast
to the plaque deficient non-recombinants.

Partially plaque purified recombinants from each
transfection were consequently characterized using
antibodies specific for the individual foreign protein
expressed. This was carried out by direct immuno-staining
of plaques on unfixed monolayers of BS-C-1 cells. As
cells were unfixed this also indicated that the target
proteins were expressed on the cell surface. For the
majority of studies rat anti-mB7-1 and B7-2 MAbs
(Pharmagen) were used to identify surface expression of
these proteins. The vaccinia recombinants were plaque
purified at least 4 times on BS-C-1 cells (African Green
monkey kidney cell line American Type Culture Collection
(ATCC Accession No. CCL 26) followed by plaquing on STO
cells embryonic mouse fibroblast, Accession No. ATCC/CRL
1503 in the presence of 6-thioguanine (TG) (Sigma Chemical
Co.) which selects against unstable single cross-over
recombinants. Recombinants were again plaque purified on
BS-C-1 cells at least once before stocks were grown in
HeLa Spinner cells. The virus was semi-purified by
ultracentrifugation through a 36% sucrose cushion.
Aliquots were prepared and stored at 80°C. A single
aliquot from each batch was thawed, sonicated and titred
on BSC-1 cells. Infected monolayers were immunostained to
determine continued expression of foreign proteins.

The B7-1 recombinant (v.MCB7.1) was further
characterized in a FACS analysis to illustrate surface
expression and its ability to bind CTLA4-Ig (B7-1 T-cell
receptor) and by Western blot analysis using the anti-B7-1
antibody.

The B7-1 recombinant has been shown to be attenuated
compared to wild-type WR, in a mouse LD^{50} assay.

Construction and Characterization of Vaccina Recombinants

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mB7-1 , mB7-2 or mHa Co-expressing Additional Genes

Additional genes were inserted into the TK region of the above recombinants. The following is a list of recombinants constructed and characterized (the first part of the code indicates the gene situated in the F13L loci under control of the E/L promoter and latter abbreviation denotes genes in the TK region):

Code	Genes Expressed and Promoters
McB7-1	mB7-1, P. E/L
McB71-1/NP	mB7-1, P. E/L Influenza NP, P7.5K
McB7-1β-gal	mB7.1, P.E/L B-gal. P.7.5K
McB7-1/ova	mB7-1, P.E/L Ovalbumin, P11.K B-gal, 11k
McB7-1/P1A	mB7-1, P.E/L Murine P1A, P7.5K β-gal. P.11k
McB7-1/B7-2	mB7-1, P.E/L B-gal, P.11k
McMHA	Measle Hemagglutin, P.E./L
McMHA/NP McMHA/β-gal	
McMHA/ova McMHA/P1A	TK inserted, details as above
McMHA/B7-2	mB7-2, 7.5K B-gal. P.11k
McB7-2	B7-2. P.E./L

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McB7-2/β-gal   B7-2, P.E./L
                      B-gal. P.11k

     A recombinant vaccinia virus containing the genes for
B7.1 and the tumor associated antigen, β-gal was deposited
with the American Type Culture Collection, 12301 Parklawn
Drive, Rockville, MD 20852 USA under Accession No. VR2485
according to terms of the Budapest Treaty. A recombinant
vaccinia virus containing the genes for IL-2 and β-gal was
deposited with ATCC under Accession No. VR2486.

     Sucrose cushion purified stocks of all characterized
recombinant viruses have been prepared.

     The above method is also employed to construct other
recombinant virus using the other previously mentioned
viruses such as fowlpox virus in combination with other
foreign antigens and immunostimulatory molecules.

Example 2

Recombinant Fowlpox Virus Expressing A TAA, LacZ and B7.1

     A recombinant fowlpox virus (rFPV), which is
replication incompetent in mammalian cells, is constructed
that express the model TAA, lacZ encoding β-gal, and the
immunostimulatory molecule, B7.1 under the influence of
the 40K vaccinia virus early/late promotor.

     The POXVAC-TC (Schering Corp.) strain of FPV is used.
FPV is propagated on primary chick embryo dermal cultures
(Jenkins, S. et al. AIDS Res. Hum. Retroviruses, 7:991,
1991). Foreign sequences are inserted into FPV by
homologous recombination as previously described (Jenkins
et al. 1991). Recombinant fowlpox contains the *E. coli*
lacZ gene and the B7.1 gene under the control of the
vaccinia virus 40K promotor (designed H6 in Rosel, J. et
al. J. Virol. 60:436, 1986), inserted into the BAM HI J
region of the FPV genome.

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